REMARKS

Claims 31-33, and 37-47 are pending. In the May 22, 2007 Action, the Examiner states that the previously indicated allowability of claims 31-33 "is not appropriate and is withdrawn or rescinded." (Paper No. 20070514). In previous papers submitted by Applicants, Applicants expressed dismay regarding the piecemeal prosecution and the pattern that the Office has exhibited in this case of reneging on indicated allowability. See particularly the Response to Office Action Including Amendment and Petition for Extension of Time, dated February 1, 2007, pages 2-3, which was submitted by Applicant's previous representative. Applicants continue to be concerned about and here maintain the objection to this disturbing trend.

In the present submission, however, Applicants wish to focus the Examiner on the reasons for which the pending claims represent patentable subject matter. As such, Applicants seek to convince the Examiner to reinstate the allowability of claims 31-32, to allow all pending claims, and to issue a Notice of Allowance. Applicants also thank the Examiner for withdrawing the rejections under 35 USC 112.

No amendments to the claims are presently submitted.

Rejection Under 35 USC 103

Claims 31-33, and 37-47 were rejected under 35 USC 103(a) as unpatentable over Cantor (US Pat. No. 5,633,003) in combination with Green (WO 96/19968). (Id. at 2).

In making the rejection, the Examiner asserted that "Cantor discloses a system for delivering a polysaccharide formulation (hyaluronic acid), that can be delivered via a route aerosol inhalation [sic] by a nebulizer...", and that "Cantor uses the same method of delivery (aerosol inhalation) for the same purpose (i.e., treating respiratory disorders) comprising a polysaccharide." (Id. at 3). The Examiner noted that "Cantor discloses that the hyaluronic acid used may be derived from bovine sources, rooster comb, human umbilical cord, or streptoccus zeopidicus." (Id.) The Examiner asserted that "[t]his implies that hyaluronic acid of different molecular weights can be used since the said sources of hyaluronic acid produces [sic] hyaluronic acid of different molecular weight." (Id.) The Examiner emphasized that "[i]n fact, the hyaluronic acid suggested by Cantor are naturally occurring hyaluronic acid ...which are known to have molecular weight of 50,000-13,000,000 daltons...", and [i]t should be noted that this molecular weight range of hyaluronic acid encompasses the molecular weight range of the hyaluronic acid claimed by applicant." (Id. at 3-4).

The Examiner acknowledged that "[t]he difference between applicants' claimed composition and the composition of Cantor is that Cantor does not disclose the concentration, molecular weight or particle size of the polysaccharide and [C]antor does not use a drug or propellant." (Id. at 4). The Examiner reiterated, however, that "Cantor suggests that hyaluronic acid from different sources ... which are known to have different molecular weights can be used..." (Id.).

To attempt to fill the acknowledged gap, the Examiner relied upon Green as "disclos[ing] an aerosol formulation for administration by inhalation containing a medicament, a sugar (a carbohydrate) and a fluorocarbon propellant for treating

respiratory disorders..." (Id.). The Examiner also asserted that Green's "medicament can include [certain] drugs..." and that "Green, like Cantor, also uses the same method of delivery (aerosol inhalation) for the same purpose (i.e., treating respiratory disorders)." (Id.)

The Examiner then concluded that "[i]t would have been obvious to one having ordinary skill in the art ... to have prepared the composition (an inhalant aerosol formulation) of Cantor comprising different concentrations, molecular weights or particle sizes of the polysaccharide in combination with a drug disclosed by Green ...and a fluorocarbon propellant to be used as an aerosol formulation for treating respiratory conditions or disorders depending on [a variety of patient-specific factors] such as the severity of the respiratory disorder or the type, age and weight of subject treated, since Cantor suggests that different molecular weights of hyaluronic acid (polysaccharide) can be used and Green disclose that drugs ... and a fluorocarbon propellant can be used as an inhalant aerosol formulation..." (Id. at 5). Also, the Examiner stated that "[o]ne having ordinary skill in the art would have been motivated, to prepare the composition (an inhalant aerosol formulation) of Cantor ...since Cantor suggests that different molecular weights of hyaluronic acid (polysaccharide) can be used and Green disclose that drugs ... can be used ..." (Id.)

The Examiner "noted that claims 38-41 appear to be free of the prior art of record. (Id. at 6) The Examiner summarily concluded, however, that "it is obvious to use other polysaccharides such including [sic] polysaccharides that are conjugated to a drug since both Cantor and Green disclose the use of polysaccharides in general." (Id.)

For the reasons set forth below, the rejection is traversed.

The Examiner mischaracterizes Cantor in concluding on page 3 of Paper No. 20070514 that Cantor "implies that hyaluronic acid of different molecular weights can be used ..." Cantor states that "forms of hyaluronic acid may be derived from bovine sources, rooster comb, human umbilical cord, or streptoccus zoepidicus..." (Cantor, Col. 3, lines 13-15). As indicated in US Patent No. 4,746,504 which was noted by the Examiner in the Action at page 4 in connection with hyaluronic acid, "[n]aturally occurring hyaluronic acid is a glycosaminoglycan consisting of a linear polymer of molecular weight of 50,000-13,000,000 daltons." Furthermore, "[i]t is a polysaccharide made of repeating units of glucuronic acid and N-acetyl-glucosamine, bound by alternating ... bonds." Naturally occurring hyaluronic acid is polydisperse and encompasses a wide range of molecular weight. For example, hyaluronic acid isolated from rooster comb may have a molecular weight spanning over the range of from 100,000 to 2,000,000 Daltons. In reporting the use of any of the naturally occurring hyaluronic acids of a given origin reported by Cantor, e.g., rooster comb hyaluronic acid, a polydisperse molecular weight range is implicated.

The claims of the present application, on the other hand, recite a polysaccharide having a molecular weight in a specified range and at a concentration of less than a recited value. Claim 31 is directed to a system comprising, among other recited elements, "a mixture comprising a polysaccharide having a molecular weight of between about 50,000 and 1.5 x 10^6 Daltons at a concentration of less than about 5.0 mg/ml (w/v) of polysaccharide, and a breathable fluorocarbon propellant". Applicants report a variety of experiments in the specification including studies using hyaluronic acid of defined molecular weight such as 227 KDa, 587 KDa, and 890 KDa.

(Specification, particularly pgs 48-53.) And, Applicants have found that the lowest molecular weight hyaluronic acid, 227 KDa, had the best properties of the three tested in terms of elastic fiber protection and optimum aerosol particle size. (Id. at 51-61). Cantor's report, on the other hand, of naturally occurring, polydisperse hyaluronic acid, provides no teaching, suggestion or motivation to one of ordinary skill in the art to achieve a system comprising a mixture having a polysaccharide of the recited molecular weight range that encompasses a polysaccharide of a defined molecular weight within the recited range.

In addition, one skilled in the art would not have considered a system adapted for delivery of a formulation to a respiratory tract of a mammal comprising a polysaccharide having a molecular weight as recited in the present claims. The art reports that low molecular weight hyaluronic acid (e.g., less than 250 KDa) may be proinflammatory. Horton M. R., et al., Induction and Regulation of Macrophage Metalloelastase by Hyaluronan Fragments in Mouse Macrophages. J. Immunol. 1999;162: 4171-4176 ("Horton"); McKee C. M., et al., Hyaluronan (HA) Fragments Induce Chemokine Gene Expression in Alveolar Macrophages. The Role of HA Size and CD44. J. Clin Invest. 1996; 98: 2403-2413 ("McKee"), which are provided in the attached Supplemental Information Disclosure Statement. In referencing previous work, Horton states that "we have shown that HA fragments with low molecular masses (200,000 Da) induce the expression of a number of inflammatory mediators including several members of the chemokine family, IL-1, TNF-alpha, IL-12, and iNOS [citations omitted]." (Horton, pg 4173, left col.) Horton reportedly studied the role of metalloproteinase (MMP) which is secreted by activated macrophages, and is a family

of enzymes that are able to degrade all components of the extracellular matrix (ECM). (Id. at pg 4171, right col.) The authors found that "low m.w. fragments induce transcription of MME in activated macrophages". (Id. at 4174, right col.; see also Abstract and pg 4174, right col. to pg 4175, end).

McKee report that "[w]e have identified a collection of inflammatory genes induced in macrophages by HA fragments but not by high molecular weight HA." (McKee, pg 1, Abstract). The authors concluded that their findings "support the hypothesis that HA fragments generated during inflammation induce the expression of macrophage genes which are important in the development and maintenance of the inflammatory response." (Id., pg 2, Abstract). As is evident from Horton and McKee, the art admonishes that low molecular weight hyaluronic acid not only generates an inflammatory response, but could likely do so by triggering more than one pro-inflammatory mechanism.

In view of the evidence of a pro-inflammatory response associated with low molecular weight hyaluronic acid reported in the art, one skilled in the art would have considered that the prior art leads away from the presently claimed invention which is adapted for delivery to a respiratory tract.

Furthermore, the presently claimed system achieves unexpected or surprising results in view of the teachings of the art. More recently published work has shown that an aerosol preparation of hyaluronic acid with a specific molecular weight of 150 KDa prevents cigarette smoke-induced emphysema in mice without inducing an inflammatory reaction in the lung. Cantor, J. O., et al., Aerosolized Hyaluronan Limits Airspace Enlargement in a Mouse Model of Cigarette Smoke-Induced Pulmonary

Emphysema. Exper Lung Res. 2005; 31: 417-430 ("Cantor 2005"), which is provided herewith as Attachment A. The authors report that "[a]s in prior studies," the results from using "aerosolized HA showed preferential binding to elastic fibers, suggesting that it may protect them from injury." (Cantor 2005, Abstract) In pertinent part to the present discussion, the authors report that "[a]lthough several studies have shown that low-molecular-weight HA may enhance the expression of cytokines [citations omitted], we observed no evidence of an inflammatory response in the HA-treated animals beyond that induced by smoke exposure." (Id. at 428).

For all of the above reasons, Cantor is insufficient, alone or in combination with Green, as a basis for making a rejection of obviousness. The use of naturally occurring polydisperse hyaluronic acid according to Cantor does not teach, suggest or provide motivation for the present system comprising a polysaccharide having a molecular weight within the recited range. Also, the presently claimed subject matter is unexpected or surprising in view of the prior art. Accordingly, the obviousness rejection has been overcome.

Applicants submit that the Examiner has mischaracterized Green in making the overreaching statement that "both Cantor and Green disclose the use of polysaccharides in general." (Id. at 6). First, Green unambiguously states, "[s]urprisingly, the applicants have now found that ***particular sugars*** may advantageously be used to prepare novel aerosol formulations." (Green, pg 1, ln 31-32) (emphasis added). Green reports that the "invention relates to aerosol formulations of use for the administration of medicaments by inhalation and in particular to a pharmaceutical aerosol formulation which comprises (a) particulate medicament; (b) ***at***

least one sugar, and (c) a fluorocarbon or hydrogen-containing chlorofluorocarbon propellant." (Green, Abstract) (emphasis added). Green states that "[t]ypical sugars which may be used in the formulations include, for example, sucrose, lactose and dextrose, preferably lactose, and reducing sugar such as mannitol and sorbitol." (Id. at pg 4, ln 22-24). Also, the Examples of Green refer to lactose, dextrose, sucrose or mannitol. The sugars reported by Green, therefore, are ***simple, single unit sugars of a molecular weight of about 180-342***. The Examiner has erroneously referred to Green as "disclos[ing] the use of polysaccharides in general." Moreover, the Examiner provides no explanation for such a far-reaching characterization.

Applicants submit that Green, whether alone or in combination with Cantor, provides no teaching, suggestion or motivation to achieve the presently claimed invention. One skilled in the art would not assume that the properties of such small sugar molecules (mol. wt. 180-342) of Green are in any way related to the strikingly larger molecules which are polysaccharides of between about 50,000 and 1.5 x 10^6 Daltons employed by Applicants. One skilled in the art would take note that Green unambiguously reported that the "particular sugars" indicated "may advantageously be used to prepare novel aerosol formulations". It would have been understood from the disclosure of Green that these simple, single unit sugars of such low molecular weight are important in the formulation of his aerosol formulations. Applicants submit that Green, therefore, would not motivate one of skill in the art to deviate from his teachings regarding the simple, single unit sugars to use in the aerosol formulation.

Moreover, Green, in reporting on the use of the "particular sugars" which are the single unit sugars of low molecular weight, tends to lead one of skill in the art away from the use of the polysaccharides as presently claimed.

Furthermore, Green is concerned with aerosol formulations for use in the administration of a drug by inhalation. (Green, pg 1, ln 3-4). In Green, a "particulate medicament" is a required element of the "aerosol formulations of use for the administration of medicaments by inhalation..." (Green, Abstract). Green reports that medicaments that can be used include a list of active agents such as "analgesics, e.g. codeine, dihydromorphine, ... anginal preparations, e.g., diltiazem; antiallergics, e.g. cromoglycate, ...antiinfectives e.g. cephalosporins...," etc. (Green, page 2, line 22-page 4, line 10, citing at page 2, line 22 – line 28). Green includes "anti-inflammatories" among the types of drugs listed (Green, page 2, lines 30-31), and also states that "[p]referred aerosol formulations" are said to include "a particulate anti-inflammatory...". (Green, page 3, line 34-36).

As noted above, the prior art taught that hyaluronic acid of low molecular weight induces a pro-inflammatory response. Green, on the other hand, indicates that the use of anti-inflammatories in his aerosol formulation is preferred. In developing a system adapted for delivery of a formulation to a respiratory tract of a mammal and in view of the preference expressed by Green for the use of anti-inflammatory medicaments, one skilled in the art would not have considered the use of a polysaccharide of the presently recited molecular weight given what was known in the art about a pro-inflammatory response resulting from the use of low molecular weight hyaluronic acid.

In addition, The Examiner has improperly cited Green with respect to **all** pending claims, although not all of the pending claims recite the presence of a drug or medicament.

It is well settled that the Examiner bears the burden to set forth a *prima facie* case of unpatentability. *In re Glaug*, 62 USPQ2d 1151, 1152 (Fed. Cir. 2002); *In re Oetiker*, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992); and *In re Piasecki*, 223 USPQ 785, 788 (Fed. Cir. 1984). If the PTO fails to meet its burden, then the applicant is entitled to a patent. *In re Glaug*, 62 USPQ2d at 1152.

When patentability turns on the question of obviousness, as here, the search for and analysis of the prior art by the PTO should include evidence relevant to the finding of whether there is a teaching, motivation, or suggestion to select and combine the documents relied on by the Examiner as evidence of obviousness. *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1731-32 (2007) (the obviousness "***analysis should be made explicit***" and the teaching-suggestion-motivation test is "***a helpful insight***" for determining obviousness) (emphasis added); *McGinley v. Franklin Sports*, 60 USPQ2d 1001, 1008 (Fed. Cir. 2001). Moreover, the factual inquiry whether to combine documents must be thorough and searching. And, as is well settled, the teaching, motivation, or suggestion to combine "***must be based on objective evidence of record***." *In re Lee*, 61 USPQ2d 1430, 1433 (Fed. Cir. 2002) (emphasis added).

Here, the rejection is devoid of evidence - or even argument - in support of the proposed combination. All that is there are conclusory remarks based upon misleading statements regarding the prior art. What the rejection should have done, but

did not, was to explain on the record ***why*** one skilled in this art would modify the disclosure of Cantor using Green to arrive at the claimed invention. As is well settled, an Examiner cannot establish obviousness by locating references which describe various aspects of a patent applicant's invention without also providing evidence of the motivating force which would impel one skilled in the art to do what the patent applicant has done. *Takeda Chem. Indus., Ltd v. Alphapharm Pty., Ltd.*, 2007 U.S. App. LEXIS 15349, *12 (Fed. Cir. June 28, 2007) (indicating that "it remains necessary to identify ***some reason*** that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound") (emphasis added); *Ex parte Levengood*, 28 USPQ2d 1300, 1301-02 (BPAI 1993). But this is precisely what the Examiner has done here. Thus, the rejection is legally deficient and should be withdrawn for this reason alone.

It is respectfully submitted that the rejection has been overcome. Reconsideration and withdrawal of the rejection are respectfully requested.

It is believed that the application is in condition for allowance. In view of the foregoing, Issuance of a Notice of Allowance is requested. If there are any questions regarding the foregoing, the Examiner is invited to call the undersigned.

I hereby certify that this correspondence is being mailed to the United States Patent Office to the Mail Stop Amendment Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450 on November 21, 2007.

Eileen M. Ebel, Reg. No. 37,316

Respectfully submitted,

By:

Eileen M. Ebel
Registration No. 37,316
BRYAN CAVE LLP
1290 Avenue of the Americas
New York, NY 10104
(P) (212) 541-2194
(F) (212) 541-4630

Attachment A

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AEROSOLIZED HYALURONAN LIMITS AIRSPACE ENLARGEMENT IN A MOUSE MODEL OF CIGARETTE SMOKE–INDUCED PULMONARY EMPHYSEMA

Jerome O. Cantor, Joseph M. Cerreta, Marcos Ochoa, Shuren Ma, Tony Chow, Gabriele Grunig, and Gerard M. Turino □ *St. John's University School of Pharmacy and Allied Health Sciences, Jamaica, New York, USA; and St. Luke's–Roosevelt Hospital Center, New York, New York, USA*

□ *This study was designed to determine if aerosolized hyaluronan (HA) could prevent airspace enlargement and elastic fiber injury in a mouse model of cigarette smoke–induced pulmonary emphysema. Compared to untreated/smoked controls, HA-treated animals showed statistically significant reductions in mean linear intercept (54 versus 65 μm; $P < .001$) and elastic fiber breakdown products (desmosine and isodesmosine) in bronchoalveolar lavage fluid (0.3 versus 7.0 ng/mL; $P < .05$). As in previous studies, the aerosolized HA showed preferential binding to elastic fibers, suggesting that it may protect them from injury. These findings support further investigation of the potential use of HA as a treatment for pulmonary emphysema.*

Keywords cigarette smoke, elastase, elastic fibers, emphysema, hyaluronan

Human pulmonary emphysema is characterized by progressive damage to the elastic fiber network of the lung, resulting in dilatation and rupture of alveoli, reduced gas exchange, and eventual respiratory failure [1, 2]. Although most approaches to the treatment of this disease have focused on the use of elastase inhibitors to prevent elastic fiber breakdown, this laboratory has developed a novel method of preventing such injury by administration of aerosolized hyaluronan (HA). Animals exposed to HA prior to intratracheal instillation of elastase had significantly less airspace enlargement than untreated controls [3–6]. The protective effect of HA may be

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Address correspondence to Jerome O. Cantor, MD, St. John's University (SAH 128), 8000 Utopia Parkway, Jamaica, New York 11439, USA. E-mail: cantorj@stjohns.edu

related to its ability to bind to lung elastic fibers, thereby preventing their degradation by elastases [3–5]. Because injury to these fibers may be a common pathway in the pathogenesis of pulmonary emphysema, this form of treatment might be effective against a number of agents that can cause the disease, including various elastases and inhaled oxidants.

To further test this concept, the current study examined the effect of aerosolized HA on mice exposed to tobacco smoke for an extended period. The smoking model presents a more stringent test of HA against a chronic form of lung injury that shares at least some of the features of the human disease. Mice were exposed to either an aerosolized solution of HA in water or water alone (controls) prior to inhaling cigarette smoke. The procedure was repeated 5 days per week over a period of 6 months. Both lung tissues and lavage fluid were examined at monthly intervals to determine if HA-treated animals were less susceptible to cigarette smoke–induced injury than controls. The results indicate that airspace enlargement was significantly reduced in animals receiving aerosolized HA, further suggesting the potential efficacy of this agent as a therapy for pulmonary emphysema.

METHODS

Experimental Design

Eight-week old female DBA/2J mice (Jackson Laboratories, ME) were divided into 3 treatment groups as follows: Group 1 was treated with HA for 1 hour prior to smoke exposure; group 2 did not receive HA prior to smoking; group 3 did not receive either HA or smoke. Groups 1 and 2 were exposed to smoke for 3 hours per day, 5 days/week, for a period of 6 months.

Exposure to HA Aerosol

Group 1 was exposed to a 0.1% solution of low-molecular-weight (150 kDa) streptococcal HA in water (Bayer, Shawnee, KS) for 1 hour, using a Misty-Ox nebulizer (Vital Signs, Totowa, NJ). Group 2 received aerosolized water alone for the same time interval. The nebulizer was connected to a heavy-duty air compressor that delivered a constant pressure of 30 psi. The aerosol entered the exposure chamber through an inflow port attached to the roof and was drawn through the chamber by negative pressure created by a vacuum pump connected to an exhaust port on the side wall. The chamber was large enough (28 × 19 × 15 inches) to permit the mice to remain in their cages while inhaling the aerosol, thereby minimizing direct handling of the animals.

Analysis of the particle size distribution of the aerosolized HA, using the same nebulizer, was performed by an independent laboratory (Teague

Enterprises, Davis, CA). These studies indicated that the mean aerodynamic diameter of the particles was less than 2 microns, which is small enough for penetration of the distal portions of the lung.

Exposure to Fluorescein-labeled HA

Mice that were not previously treated with either HA or cigarette smoke were exposed to low-molecular-weight (150 kDa) fluorescein-labeled HA (Bayer, Shawnee, KS) for 1 hour, then asphyxiated with CO_2 either 1 hour or 24 hours following exposure. Their lungs were inflated in situ with 10% neutral-buffered formalin at a constant pressure of 20 cm water. After a period of 2 hours, the lungs and heart were removed and fixed for an additional period of several days in formalin. The extrapulmonary structures were then dissected off and the lung tissues were randomly cut and entirely submitted for histological processing. Unstained, deparaffinized slide sections were examined with a fluorescence microscope.

Exposure to Cigarette Smoke

Following administration of either aerosolized HA or water, the nebulizer was disconnected and the smoking machine (Model TE-10; Teague Enterprises, Davis, CA) was attached to the exposure chamber. Both treatment groups were exposed to cigarette smoke for a period of 3 hours a day, 5 days per week. The smoking machine simultaneously burned 2 filtered research-grade cigarettes (type 2R4F, University of Kentucky). Each cigarette was puffed once per minute for 2 seconds at a flow rate of 1.05 L/min, yielding 35 cc of smoke. This cycle was repeated 9 times before ejecting the cigarette and loading a new one. Proper flow rate was maintained by a vacuum pump, which established negative pressure at the exhaust port. Total smoke particulates averaged 91 mg/m^3.

Light Microscopic Studies

Two to 6 months following initiation of smoke exposure, mice were asphyxiated with CO_2 and their lungs were inflated in situ with 10% neutral-buffered formalin at a constant pressure of 20 cm water (untreated/unsmoked animals were also sacrificed at the start of the experiment). After a period of 2 hours, the chest contents were removed and fixed for several days in formalin. The extrapulmonary structures were then dissected off and the lung tissues were randomly cut into smaller fragments (approximately 8) and entirely submitted for histological processing. Slide sections stained with hematoxylin and eosin were coded and examined with the light microscope to determine histological changes and to quantify

airspace diameter by the mean linear intercept method [7]. For each animal, at least twenty high-power fields were measured by an experienced morphologist (JMC). Additional sections were treated with the Verhoeff–Van Gieson stain to identify elastic fibers.

Bronchoalveolar Lavage Cytology

Mice were asphyxiated with CO_2 and their lungs were lavaged 3 times with 1-mL aliquots of Hanks' solution. Cells were separated from the recovered fluid and counted with a Neubauer chamber. Morphologic evaluation was performed with cytocentrifuge preparations stained with Wright-Giemsa solution.

Measurement of Desmosine and Isodesmosine

The levels of the elastin-specific, cross-linking amino acids, desmosine and isodesmosine, were measured in both bronchoalveolar lavage fluid and whole lung tissues. To separate these amino acids from elastin peptides, both cell-free lavage fluids and homogenized lung tissues were hydrolyzed in 6 N HCl at 110°C for 24 hours. The hydrolysates were then filtered and evaporated to remove acid. Desmosine and isodesmosine were quantified by high-performance liquid chromatography and electrospray ionization mass spectrometry, according to previously published procedures [8].

Data Analysis

The Newman-Keuls multiple comparison test was used to determine statistically significant differences ($P < .05$) among the 3 treatment groups. The 2-sample t test and Mann-Whitney rank-sum test were used to determine statistical significance when comparing only 2 groups.

RESULTS

Lung Histology

Exposure to tobacco smoke resulted in the presence of large numbers of carbon-laden macrophages in the lungs of both the untreated/smoked and HA-treated/smoked animals. These cells were mostly present in the pulmonary interstitium and were not accompanied by alveolitis or fibrosis.

The lungs of both the untreated/smoked and HA-treated/smoked animals also contained peribronchial lymphohistiocytic infiltrates that extended into the lamina propria (Figure 1). Prominent papillary

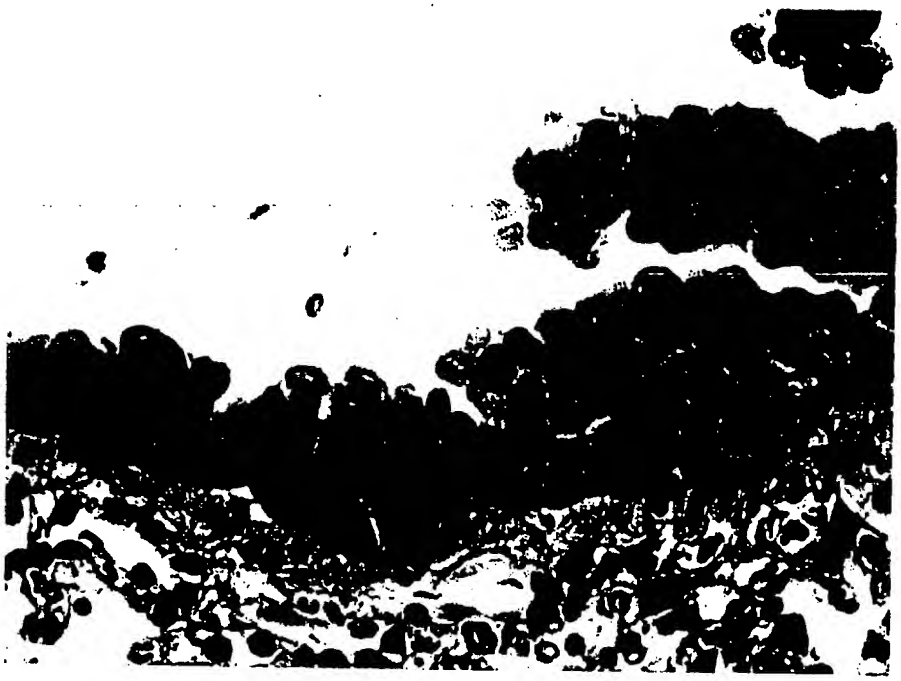


FIGURE 1 Section of bronchial wall from an untreated, smoked mouse after 6 months of smoke exposure. Papillary hyperplasia of the bronchial epithelium is noted along with a surrounding lymphohistiocytic infiltrate that extends into the lamina propria. Original magnification: ×1200 (hematoxylin and eosin).

hyperplasia of the bronchial epithelium was also noted, including an accumulation of elastic fibers within the underlying connective tissue stalks (Figure 2).

After 3 months of smoke exposure, moderate emphysematous changes were present in the lungs of the untreated/smoked animals (Figure 3*A*). In contrast, only minimal airspace enlargement was observed in the lungs of the HA-treated/smoked group over the entire course of the experiment (Figure 3*B*).

None of the animals in the study succumbed to the cigarette smoke, whether administered alone or in combination with aerosolized HA.

Deposition of Fluorescein-labeled HA in The Lung

Fluorescence microscopy revealed a rapid influx of labeled HA into the lung. One hour following termination of exposure to fluorescein-labeled HA, a pattern of linear fluorescence was seen in association with interstitial, pleural, and vascular elastic fibers (Figure 4). At 24 hours, elastic fiber fluorescence was still present, although diminished in intensity, consistent with gradual clearance of the exogenous HA from the lung.

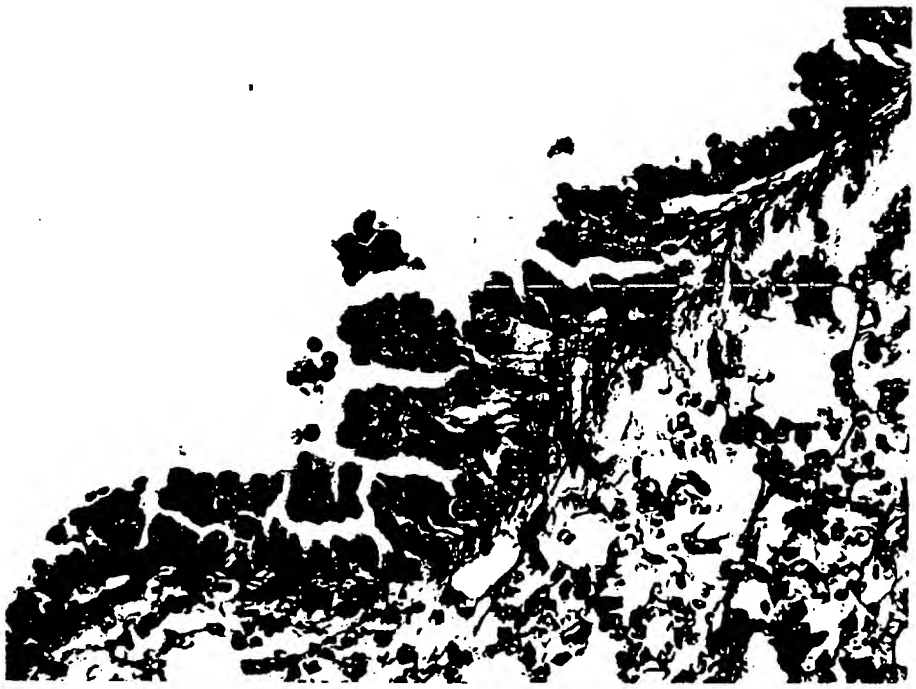


FIGURE 2 Accumulation of elastic fibers is seen in association with the bronchial papillary hyperplasia (*arrow*). Original magnification: ×600 (Verhoef–Van Gieson stain).

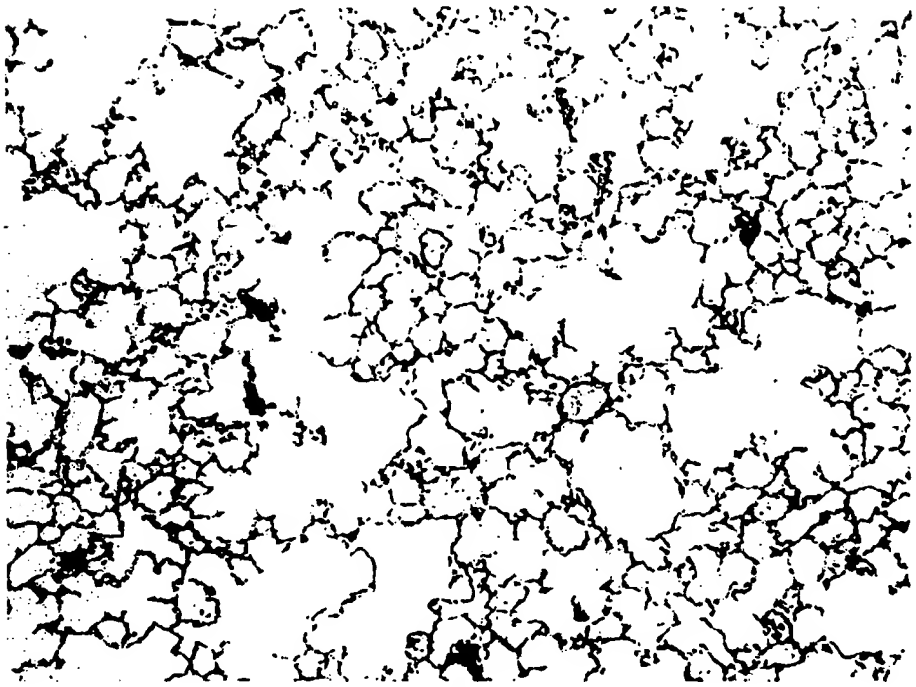
Mean Linear Intercept Determination

Treatment with aerosolized HA significantly decreased smoke-induced airspace enlargement (Figure 5). The mean linear intercepts of the HA-treated/smoked and untreated/smoked groups respectively were 47 μm versus 58 μm at 2 months ($P < .01$), 50 μm versus 66 μm at 3 months ($P < .05$), 58 μm versus 68 μm at 4 months ($P < .001$), 57 μm versus 68 μm at 5 months ($P < .01$), and 55 μm versus 63 μm at 6 months.

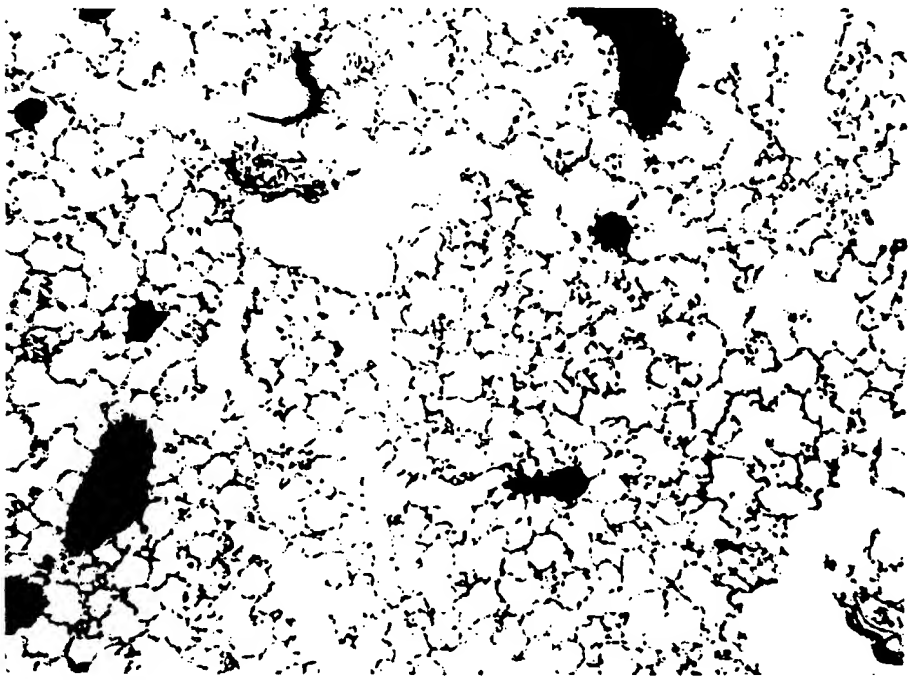
Most of the airspace enlargement in both groups occurred during the first few months. The mean linear intercepts of the untreated/smoked and HA-treated/smoked groups did not increase significantly after 2 and 4 months, respectively.

Interestingly, the mean linear intercept of the untreated/unsmoked animals increased from 41 μm at the beginning of the experiment to 59 μm at 5 months ($P < .0001$). The airspace enlargement included mild to moderate emphysematous changes (Figure 6), which may have resulted from increased susceptibility to oxidant injury in this particular strain of mice [9].

The mean linear intercept of the untreated/unsmoked mice nevertheless remained significantly lower than that of the untreated/smoked group at both 4 months (58 μm versus 68 μm; $P < .001$) and 5 months (59 μm



(A)



(B)

FIGURE 3 (*A*) Airspace enlargement is clearly evident in a lung exposed to cigarette smoke for 3 months without HA treatment. (*B*) In contrast, only minimal airspace enlargement is seen in a lung exposed to cigarette smoke for 3 months with HA treatment. Original magnification: ×60 (hematoxylin and eosin).

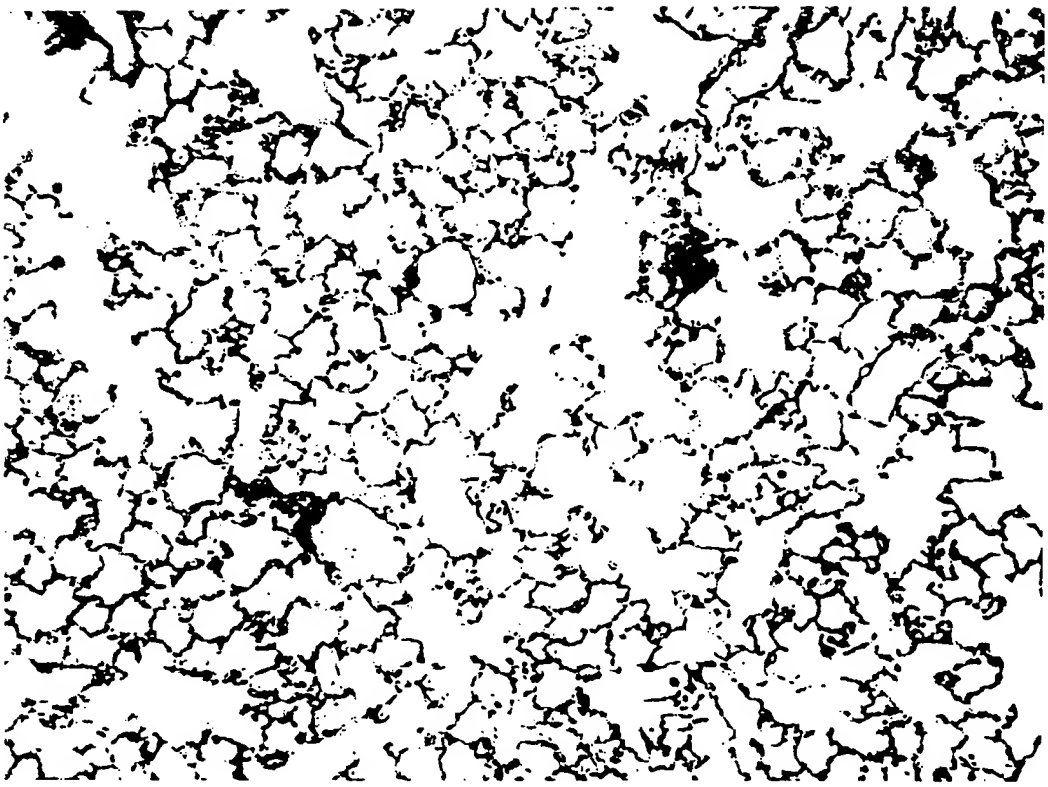


FIGURE 6 At 5 months, the lungs of the untreated/unsmoked animals showed mild to moderate emphysematous changes. Original magnification: ×60 (hematoxylin and eosin).

suggesting the possibility that hyaluronidase may facilitate the breakdown of these fibers by making them more accessible to injury. This hypothesis was also supported by studies demonstrating that pretreatment of the lung with hyaluronidase enhances airspace enlargement induced by intratracheal administration of elastase [6, 11].

Experiments were then undertaken to examine the effect of HA itself on this model of emphysema. Animals treated with an aerosol composed of 0.1% HA in water for 50 minutes prior to intratracheal instillation of elastase had significantly less airspace enlargement than controls treated with aerosolized water and elastase [3, 4].

The exact mechanism by which HA prevents lung injury is not yet well understood. HA does not directly inhibit elastases, but instead appears to bind to elastic fibers and prevent elastases from attacking them [3–6]. Studies using aerosolized fluorescein-labeled HA demonstrated preferential adherence of the polysaccharide to lung elastic fibers [4, 5]. HA has also been shown to bind to elastic fibers in vitro and prevent elastolysis by several different types of elastase, including human metalloproteinase, which may be responsible for emphysematous changes associated with cigarette smoking [3].

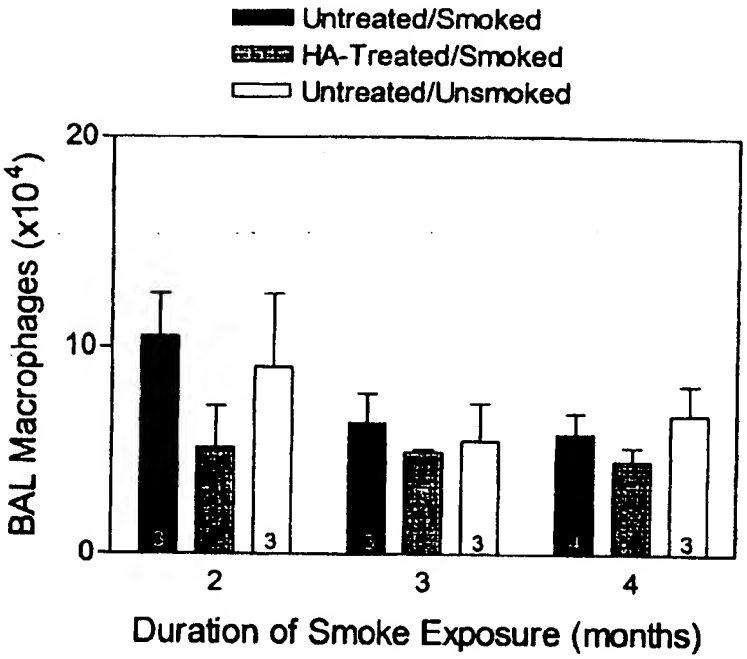


FIGURE 7 Measurements of the total number of lavaged macrophages did not reveal statistically significant differences among the 3 groups at individual time points, although the cumulative (2- to 4-month) mean of the HA-treated/smoked group was significantly lower than that of the untreated/smoked group. The number of animals tested is indicated within the bars. T-bars denote SEM.

The interaction between HA and elastic fibers may possibly involve formation of electrostatic or hydrogen bonds. Such binding sites may not be situated on the elastin protein itself, but may instead involve the surrounding microfibrillar component or other glycoproteins. The exogenously administered HA might also combine with native HA that is normally in close proximity to elastic fibers [12]. Because HA self-aggregates, it may form large molecular complexes that provide a protective barrier against both free elastases and the cells that secrete them [13].

Although smoke exposure is associated with elastic fiber degradation [14], neither the HA-treated nor untreated animals exposed to smoke showed a significant reduction in total lung desmosine and isodesmosine content compared to the untreated/unsmoked group. This finding may be at least partially explained by the proliferation of elastic fibers in the bronchial walls of the smoke-exposed mice. Similar findings have been noted in human lungs exposed to cigarette smoke and may reflect an ongoing repair process in response to chronic smoke exposure [15].

The fact that the HA-treated/smoked animals had a significantly lower mean linear intercept than the untreated/unsmoked group at 2 months raises the question of whether HA may be inhibiting normal lung growth. Although this possibility cannot be completely ruled out, it is more likely that HA is

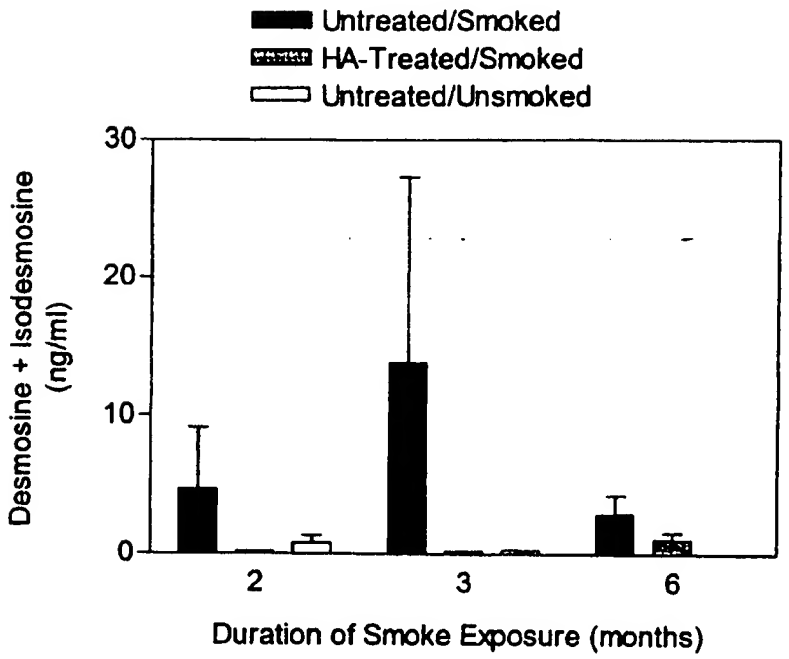


FIGURE 8 The levels of desmosine and isodesmosine in lavage fluid showed marked variation in the untreated/smoked animals at 2 and 3 months, but the means were nevertheless much higher than those of the other treatment groups. The cumulative mean of the untreated/smoked animals was significantly increased compared to that of the HA-treated/smoked group ($P < .05$). $N = 3$ for each bar (no data for untreated/unsmoked group at 6 months). T-bars denote SEM.

mainly preventing elastic fiber injury, because the airspace enlargement in the untreated/unsmoked group did not simply reflect normal lung maturation, but was instead associated with emphysematous changes.

The leveling off of airspace enlargement in the untreated/smoked group after several months is consistent with an adaptive response to chronic smoke exposure. A number of studies suggest that enhanced synthesis of endogenous antioxidants may limit the damaging effects of tobacco smoke and other oxidants [16–20]. Furthermore, changes in the interstitial extracellular matrix resulting from continual injury and repair (e.g., increased collagen content) could decrease the likelihood of alveolar wall rupture due to elastase activity or mechanical stress.

With regard to the potential toxicity resulting from prolonged exposure to HA, it should be noted that this agent has been administered to other tissues without adverse consequences [21–23]. Although several studies have shown that low-molecular-weight HA may enhance the expression of cytokines [24, 25], we observed no evidence of an inflammatory response in the HA-treated animals beyond that induced by smoke exposure.

Because elastic fiber breakdown may be a final common pathway in pulmonary emphysema, HA might be effective against a number of agents

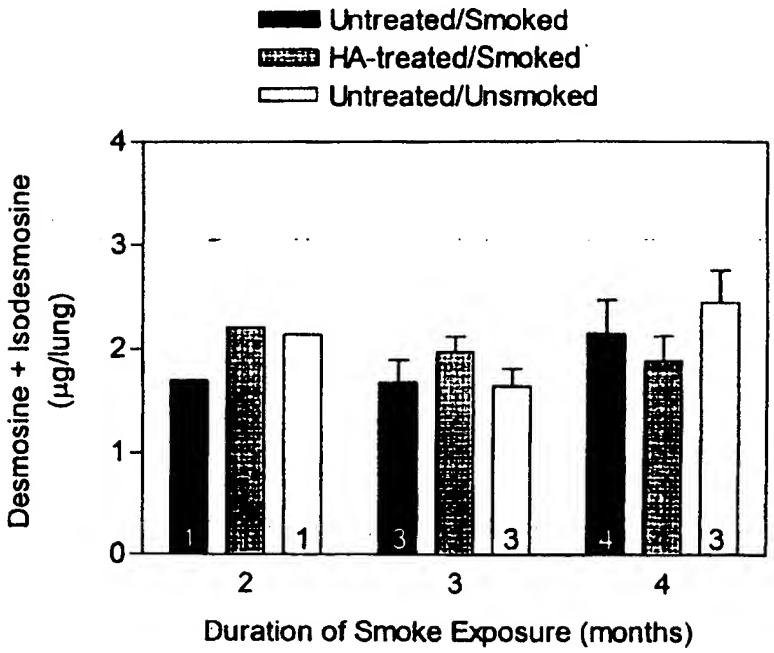


FIGURE 9 Measurements of total lung desmosine and isodesmosine did not reveal any statistically significant differences among the 3 treatment groups. The number of animals tested is indicated within the bars. T-bars denote SEM.

capable of causing the disease. In contrast to other proposed treatments, such as specific elastase inhibitors, HA might provide broader protection of the lung with fewer potential side effects. The generally slow progression of pulmonary emphysema suggests that even a small decrease in the rate of elastic fiber injury could significantly lower the risk of respiratory failure in patients with this disease.

REFERENCES

[1] Barnes PJ, Shapiro SD, Pauwels RA: Chronic obstructive pulmonary disease: molecular and cellular mechanisms. Eur Respir J. 2003;22:672–678.

[2] Shapiro SD: Proteinases in chronic obstructive pulmonary disease. Biochem Soc Trans. 2002;30:98–102.

[3] Cantor JO, Shteyngart B, Cerreta JM, Armand G, Liu M, Turino GM: The effect of hyaluronan on elastic fiber injury in vitro and elastase-induced airspace enlargement in vivo. Proc Soc Exp Biol Med. 2000;225:65–71.

[4] Cantor JO, Cerreta JM, Armand G, Turino GM: Aerosolized hyaluronic acid decreases alveolar injury induced by human neutrophil elastase. Proc Soc Exp Biol Med. 1998;217:471–475.

[5] Cantor JO, Cerreta JM, Armand G, Turino G.M: Further investigation of the use of intratracheally administered hyaluronic acid to ameliorate elastase-induced emphysema. Exp Lung Res. 1997; 23:229–244.

[6] Cantor JO, Cerreta JM, Keller S, Turino GM: Modulation of airspace enlargement in elastase-induced emphysema by intratracheal instillment of hyaluronidase and hyaluronic acid. Exp Lung Res. 1995;21:423–436.

[7] Dunnill MS: Quantitative methods in the study of pulmonary pathology. Thorax. 1962;17:320–328.
[8] Ma S, Lieberman S, Turino GM, Lin YY: The detection and quantitation of free desmosine and isodesmosine in human urine and their peptide-bound forms in sputum. Proc Nat Acad Sci USA. 2003;100:12,941–12,943.
[9] Hele D: Meeting report: First Siena international conference on animal models of chronic obstructive pulmonary disease. Respir Res. 2002;3:12.
[10] Cantor JO, Cerreta JM, Armand G, Keller S, Turino GM: Pulmonary air-space enlargement induced by intratracheal instillment of hyaluronidase and concomitant exposure to 60% oxygen. Exp Lung Res. 1993;19:177–192.
[11] Murakami H, Yoshida M, Aritomi T, Shiraishi M, Ishibashi M, Watanabe K: Effects of hyaluronidase on porcine pancreatic elastase-induced lung injury. J Jpn Respir Soc. 1998;36:577–584.
[12] Baccarani-Contri M, Vincenzi D, Cicchetti F, Mori G, Pasquali-Ronchetti I: Immunocytochemical localization of proteoglycans within normal elastin fibers. Eur J Cell Biol. 1990;53:305–312.
[13] Scott JE, Cummings C, Brass A, Chen Y: Secondary and tertiary structures of hyaluronan in aqueous solution, investigated by rotary shadowing-electron microscopy and computer simulation. Hyaluronan is a very efficient network-forming polymer. Biochem J. 1991;274:699–705.
[14] Ofulue AF, Ko M, Abboud RT. Time course of neutrophil and macrophage elastinolytic activities in cigarette smoke-induced emphysema. Am J Physiol. 1998;275:L1134–L1144.
[15] Carroll NG, Perry S, Karkhanis A, Harji S, Butt J, James AL, Green FH: The airway longitudinal elastic fiber network and mucosal folding in patients with asthma. Am J Respir Crit Care Med. 2000;161:244–248.
[16] Comhair SA, Erzurum SC: Antioxidant responses to oxidant-mediated lung diseases. Am J Physiol Lung Cell Mol Physiol. 2002;283:L246–L255.
[17] MacNee W: Oxidants/antioxidants and COPD. Chest. 2000;117:303S–317S.
[18] Baskaran S, Lakshmi S, Prasad PR: Effect of cigarette smoke on lipid peroxidation and antioxidant enzymes in albino rat. Indian J Exp Biol. 1999;37:1196–2000.
[19] Weller BL, Crapo JD, Slot J, Posthuma G, Plopper CG, Pinkerton KE: Site- and cell-specific alteration of lung copper/zinc and manganese superoxide dismutases by chronic ozone exposure. Am J Respir Cell Mol Biol. 1997;17:552–560.
[20] Wurzel H, Yeh CC, Gairola C, Chow CK: Oxidative damage and antioxidant status in the lungs and bronchoalveolar lavage fluid of rats exposed chronically to cigarette smoke. J Biochem Toxicol. 1996;10:11–17.
[21] Graf J, Neusel E, Schneider E, Niethard FU: Intra-articular treatment with HA in osteoarthritis of the knee joint: a controlled clinical trial versus mucopolysaccharide polysulfuric acid ester. Clin Exp Rheum. 1993;11:367–372.
[22] Goldberg EP, Burns JW, Yaacobi Y: Prevention of postoperative adhesion by precoating tissues with dilute sodium hyaluronate solutions.Prog Clin Biol Res. 1993;381:191–204.
[23] King SR, Hickerson WL, Proctor KG: Beneficial actions of exogeneous hyaluronic acid on wound healing. Surgery. 1991;109:76–84.
[24] Mascarenhas MM, Day RM, Ochoa CD, Choi WI, Yu L, Ouyang B, Garg HG, Hales CA, Quinn DA: Low molecular weight hyaluronan from stretched lung enhances interleukin-8 expression. Am J Respir Cell Mol Biol. 2004;30:51–60.
[25] McKee CM, Penno MB, Cowman M, Burdick MD, Streiter RM, Bao C, Noble PW: Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. J Clin Invest. 1996;98:2403–2413.